Method of calibration of reverse transcription using a synthetic messenger RNA (SmRNA)

The invention pertains to the field of reverse transcription reactions and relates to a new method of calibration with a synthetic messenger RNA (SmRNA).

Quantitative RT-PCR assays involve simultaneously amplifying control molecules and samples containing a target mRNA. Known amounts of a control molecule are thermally cycled with tubes, wells or slides, containing the unknown amount of target. In addition to the pair of PCR primers for the target, a pair of PCR primers is required for the control molecule. Following amplification, the amounts of amplified products are compared. However, inherent variation in amplification efficiency among primers results in poor quality of results.

More recently, this method has been improved. Kits comprising fluorescent dyes and quenchers are provided by Applied Biosystems and are available under the trademark TaqMan™. Improvements allowed the development of real-time quantitative PCR.

These methods have been reviewed for example in Heid et al, Real-time quantitative PCR. Genome Research. 1996; 6: 986-994, and in Gibson et al, A novel method for real time quantitative RT-PCR Genome research. 1996; 6 : 995-1001. Despite the use of different sets of tubes containing triplicate two-fold dilutions of a control molecule and a fixed amount of unknown target, variation in the quantification between samples still occurs.

We found in connection with the invention that such drawback is due to the nature of the control molecule used. In Heid et al, the internal standard used for normalization is an endogenous gene such as β-actin. Endogenous genes are not appropriate since they may vary from one sample to the other and can interfere with amplification of related gene sequences. In Gibson et al, it is proposed to use a random sequence selected from the target sequence or a modified sequence deriving

from the target sequence. In this case, variation between sample may also occur and it would require the design of one control molecule for each target sequence, which makes it impossible when analyzing the expression of large number of genes.

In Wang et al, Quantification of mRNA by the polymerase chain reaction, Proc. Natl. Acad. Sci. USA, 1989, 86 : 9717-9721, it is suggested to use a standard whose sequence comprise complementary sequence of the target gene so that one pair of primer is sufficient to amplify both products. Again, such approach is irrelevant with the analysis of a large number of genes. A similar method is described in WO 00/20629.

In these competitive quantitative RT-PCR methods, PCR primers sequences for the standard are strictly identical to the PCR primers of the target mRNA.

There are mainly two alternatives regarding the standard:

- Its sequence is homologous to the mRNA internal sequence and will be referred as "competitive homologous external standard". It is also commonly called "internal standard". Such internal standard enters in competition with the target mRNA in course of the PCR reaction (see for example Gibson et al, Genome Research, vol. 6 No 10, Oct. 1996 p. 995-1001; WO 91/02817; WO 01/16367; Siebert et al, Nature, vol. 359, Oct. 1992, p. 557-558; Zamorano et al, Neuroendocrinology, vol. 63, 1996, pages 397-407; Jensen et al, Journal of Immunological Methods, vol. 215, No 1-2, June 1998, p. 45-58; Sheflin et al, Endocrinology, Vol. 132, No 6, 1993, p. 2319-2324.

- Its sequence is homologous to the target mRNA only or mainly for the segment corresponding to the primers. In this case, the standard is called "competitive heterologous external standard". Still, when such standard is added to the reaction mixture, it competes with the amplification of the target mRNA; WO 91/02817.

In all the above cases, a standard sequence homologous the target mRNA sequence or an standard heterologous sequence with a similar size and G+C content compared to the target is used to achieve a same reverse transcription efficacy between the standard and the target. But, the analysis requires a large series of

reactions with different ratios between the competitive standards and the targets. This is not suitable with high through-put quantification of different transcripts of one sample; Bustin, Journal of Molecular Endocrinology (2000) 25 :169-193 ; p. 173 lignes 44-51.

Furthermore, homologous standard are usually shorter than the target mRNA. Considering that the reverse transcription efficacy seems to be dependent more likely to the length rather than to the composition of the RNA (Figure 17), we do not believe that a short homologous standard RNA can be used to determine with precision the reverse transcription efficacy of its target mRNA.

Because quantitative PCR amplifications (real-time PCR) is now widely used for the quantification of the relative alteration in mRNA expression, controlling the RT reaction efficiency using real-time PCR has become an important factor before the further use of the RT products (microarrays, DD-RTPCR ...).

We provide a solution to address the above mentioned problems based on the use of a synthetic messenger RNA (SmRNA) designed for normalization of reverse transcription reaction of mRNAs of a biological sample, which SmRNA does not interfere with the reverse transcription of endogenous mRNA of said sample. Therefore, we provide a new method which is the opposite of the above mentioned prior art competitive methods. Indeed, the method of the invention is based on a non-competitive approach. The goal is not to assess the absolute quantity of a mRNA in a sample. In other words, the invention do not seek to design a standard for a specific target mRNA. Rather, the purpose of the invention is to determine the reproducibility of the reverse transcription of any mRNA using a non-competitive standard. Normalization of the reverse transcription between samples is achieved by calculating the efficacy of the reverse transcription of the SmRNA.

The standard of the invention is a non-competitive heterologous external standard referred as "SmRNA" and is particularly advantageous for the analysis of different genes from different samples using non-competitive real-time quantitative RT-PCR.

Description

Therefore, in a first aspect, the invention relates to a method of quantifying and normalizing products of reverse transcription reaction of mRNA extracted from a biological sample, wherein said method comprises the steps consisting of adding a synthetic messenger RNA (SmRNA) which does not compete with the reverse transcription of target mRNAs of said sample to a reaction mixture comprising mRNA extracted from said sample.

More particularly, the method comprises the steps of :

a) adding a synthetic messenger RNA (SmRNA) which does not compete with the reverse transcription of target mRNAs of said sample and which do not interfere with the reverse transcription of endogenous mRNA of said sample to a reaction mixture comprising mRNA extracted from said sample;

b) determining the reverse transcription efficacy ρ for the smRNA and,

c) adjusting the level of target mRNA transcripts by multiplication with ρ.

Preferably, said SmRNA does not interfere with the reverse transcription of any mRNA of said sample. This is to say that SmRNA displays less than 95%, 97% or 99% or preferably less than 99,50 % identity with the biological sequence of the sample, such as a human sequence, but more generally such as a mammalian sequence, a plant, a bacteria and fungi sequence.

In this method, the reverse transcription efficacy ρ for the smRNA is calculated and is used has a parameter for normalizing reverse transcription of mRNA of different samples, wherein the number of endogenous mRNA transcripts is multiplied by ρ.

For example, the addition of 4000 to 65 000 000 copies of SmRNA in 500 ng total RNA allowed to calculate that SmRNA reverse transcription efficacy ρ is about 10% (see Figure 14). ρ is determined by linear regression analysis following $y = (1+\rho) x - C$; wherein x is the quantity of SmRNA and y is the quantity of sCDNA (figure 14).

In frame with the invention, "percent identity" means the percentage of identical nucleotide between two sequences when compared according the best alignments, this percentage being statistical and the differences between the two sequences being at random and on the total length of the sequences. Optimal alignments of sequences can be achieved by means of the algorithm of Smith Waterman (1981) [Ad. App. Math. 2 : 482], with the algorithm for local homology described in Neddleman and Wunsch (1970) [J. Mol. Biol. 48 : 443], with the similarity search method described by Lipman (1988) [Proc. Natl. Acad. Sci. USA 85 : 2444], with softwares using GAP, BESTFIT, FASTA and TFASTA algorithms available in Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI and DNASIS,Version 2.5 for Windows; Hitachi Software Engineering Co., Ltd, South San Francisco, CA, by using the standard parameters described in the manufacturer brochure.

Another possibility is to use the BLAST or FASTDB programs available at WWW.ncbi.nlm.nih.gov with the following parameters "Mismatch penalty 1.00; Gap Penalty 1.00; Gap Size Penalty 0.33; joining penalty 30.0. These algorithms are displayed in .Current Methods in Sequencing and synthesis Methods and Applications, pages 127-149, 1988, Ala R. Liss, Inc".

Another feature to define SmRNA of the invention is the fact that it does not hybridize to any mRNA of a given sample in high stringent conditions (for example hybridization in a mixture containing 5 x SSPE, 5x Denhart solution, 0.5% SDS (w/v) and 100 μg/ml salmon sperm DNA].

Said SmRNA allows to perform a reverse transcription reaction and to calibrate the quantity of each target mRNA obtained after amplification with the quantity of the cDNA amplified corresponding to said SmRNA without competition with the target mRNAs and preferably without interfering with reverse transcription of any mRNA of the sample. For example, the addition of 50 119 to 501 000 000 copies of SmRNA to 500 ng total ARN does not interfere with reverse transcribed mRNA coding for GAPDH or BDNF (Figure 15). The addition of $50x10^6$ copies of SmRNA to 100 ng (1 UA), 250 ng (2.5 UA) or 500 ng (5 UA) total RNA does not

interfere with reverse transcribed mRNA of GAPDH (1 UA ; 2.5 UA or 5 UA), since the slope of correlation line is ~1.0 (Figure 16).

In this method, said SmRNA can be added in different reaction mixtures comprising one sample in different dilutions.

Said SmRNA may further comprise a polyA segment. This allows to perform the reverse transcriptase reaction using a polydT primer for both the target mRNAs and the SmRNA.

The SmRNA may be about 80 to 150 nucleotide long or longer, preferably about 100 nucleotide long and comprise particular sequences so as to be specifically amplified with a pair of primers designed to avoid primer dimerization. Short SmRNA is preferred because reverse transcription efficacy is greater for short than for longer RNA (Figure 17). And the greater the reverse transcription efficacy is, the more reliable will be both its determination and the normalization of the efficacy between samples.

The method depicted above may consist of quantitative RT-PCR, DD-RT-PCR, eventually perform in connection with macro or micro-arrays, wherein quantification of target mRNAs is performed by means of normalization with said SmRNA. The invention can be practiced on DNA microarrays. In this case, the cDNA corresponding to the SmRNA is amplified and spotted onto said microarrays. This is an important advantage of the said mRNA, since a same mRNA sequence can be used first to help calibrating the RT reaction in microarrays, and second, to calibrate the RT reaction in real-time RT-PCR (which is a commonly used technique employed following microarrays to validate the results obtained). In other words, both techniques (microarrays and real-time RT-PCR) can use a same standard to control the reaction's efficiency.

Alternatively, the method of the invention may consist of Northern blotting wherein said SmRNA is pooled with samples. Furthermore, the SmRNA may be used to calibrate the antisense RNA amplification method (Philips and Eberwine,

"Antisense RNA amplification: a linear amplification method for analyzing the mRNA population from single living cells", Methods, Vol. 10 (1996), pp. 283-288) wherein said SmRNA is pooled with reaction mixture.

In a particular embodiment, the SmRNA consists of a SEQ ID No 1, SEQ ID No 2, or sequences deriving thereof. It is reverse transcribed into "ScDNA" of SEQ ID No 3 or SEQ ID No 4, preferably using a pair of primer consisting of SEQ ID No 5 and SEQ ID No 6. Such primers are optimized so as to avoid dimerization and are specific for the SmRNA sequence. These SmRNA present a unique sequence to our knowledge when performing a sequence search in Genebank for example. The same applies for the SEQ ID No 3 to 6.

In a second embodiment, the invention relates to a synthetic messenger RNA (SmRNA) designed for normalization of reverse transcription reaction of mRNAs of a biological sample, wherein said SmRNA does not compete with target mRNAs and does not interfere with the reverse transcription of endogenous mRNAs of said sample, which SmRNA displays less than 95% or 99% identity with any biological sequence, comprises a poly A segment and is about 80 nucleotide long or longer, preferably about 100 nucleotide long. In other words, the SmRNA referred herein above does not interfere with the reverse transcription of endogenous mRNA of said sample in the sense that it does not hybridize under stringent conditions to any mRNA sequence of the sample.

This SmRNA may further comprise at least two segments which are complementary to at least two primers that are designed to avoid primer dimerization.

One preferred SmRNA has the sequence shown in SEQ ID No 1 or SEQ ID No 2. Another feature of these particular SmRNA is two segments which are complementary to two primers of SEQ ID No 5 and SEQ ID No 6.
In this regards, the invention is also directed to a primer or probe selected from of SEQ ID No 5 and SEQ ID No 6.

The invention also encompasses a cDNA obtained from a reverse transcription reaction of a SmRNA as defined above. One particular cDNA consists of SEQ ID No 3 or SEQ ID No 4.

SmRNAs of the invention may also comprise a segment which is homologous to the target mRNA and are devoid of sequences complementary to the primers sequences used to amplify the target mRNA. Sequences downstream and upstream of this SmRNA can be added for amplifying the cDNA corresponding to the SmRNA (ScDNA) (SEQ ID n° 5 and 6 for example). In this regards, the sequence of the SmRNA will be for the most part homologous to the target mRNA sequence, but this SmRNA will not compete during PCR since primers for the ScDNA and target cDNA are different.

In another embodiment, the invention concerns a vector comprising a sequence encoding the SmRNA of the invention. The term "vector" refers to a DNA molecule originating from a virus, a bacteria, or the cell of a higher organism into which another DNA fragment of appropriate size can be integrated without loss of the vectors capacity for self-replication; a vector introduces foreign DNA into host cells, where it can be reproduced in large quantities. Examples are plasmids, cosmids, and yeast artificial chromosomes; vectors are often recombinant molecules containing DNA sequences from several sources.

Preferred vectors are plasmids, more specifically plasmids which comprise the construct as shown in figure 1, notably the sequence SED ID No 7 or SEQ ID No 9 and 11. The invention also relates to a vector as depicted in figure 2. More particularly, the invention is directed to a vector as mentioned above which further comprise any sequence corresponding to SEQ ID No 9, 10 and 11 preceded by a sequence corresponding to a RNA polymerase promoter, for example RNA polymerase T7 promoter (sequence ID No 8) and referred as to "DNA probe DNAΣ" (see figure 3).

In still another embodiment, the invention is aimed at a kit for quantification of mRNAs of a biological sample comprising a SmRNA, a vector or a DNA probe as defined above. Such kit may further comprise primers of SEQ ID No 5 and 6.

The invention also relates to the use of a SmRNA, a vector or a DNA probe as defined above for calibrating target mRNAs during quantification in RT-PCR reaction, more particularly in frame with Q-RT-PCR or DD-RT-PCR, which may optionally be practiced with DNA microarrays.

The invention also relates to the use of a SmRNA, a vector or a DNA probe as defined above for calibrating target mRNAs in Northern blot analysis or for calibrating antisense RNA amplification method.

The invention is further illustrated in the examples below.

EXAMPLE 1: Calibration of the reverse transcription using a synthetic messenger RNA (SmRNA) obtained from a synthetic DNA probe (DNAΣ)

1.1 Characteristics of the DNA probe: DNAΣ

- Name of the DNA probe : DNAΣ
- Total Length : 182 bp
- Insertion of a restriction site for BSM I allowing the linearization of the vector just after the poly A sequence
- Presence of the T7 promoter allowing the in vitro transcription of a synthetic messenger RNA (SmRNA)

1.2 Construction of the DNA probe

- Insertion of a 101 bp fragment (shown below) in the pGEM®-T Easy Vector with a T4 DNA ligase (Promega).

5'TTCTTCGACTCACTGCAGACTACTGATGGAATGACGTAGTACGAATAC TCGACTGGTCTCAACATGAAAAAAAAAAAAAAACGCATTCAACCTGTCTG ACTA 3' (SEQ ID No 11).

- Linearization of the cloned plasmid with the Aat II and Sph I restriction enzymes (New England BioLabs®Inc).

3- Insertion of a 27 bp fragment (shown below) with a T4 DNA ligase (Promega).

5'-CGGGACAAGAAGGTGGAAGACGTCATG-3' (SEQ ID No 9).

The resulting plasmid referred as pGEM®-DNAΣ is shown in Figure 2.

1.3 Synthesis of the DNA probe "DNAΣ"

PCR amplification of the plasmid pGEM®-DNAΣ using:

- a mix of HotStarTaq DNA polymerase (Qiagen) and ProofStar DNA polymerase (Qiagen).
- Forward primer A containing the T7 promoter: 5'TAATACGACTCACTATAGGGCGGGACAAGAAGGTGGAAG3' (SEQ ID No 12)
- Reverse primer A:

5'TAGTCAGACAGGTTGAATGCG3' (SEQ ID No 13)

Sequence of the DNA probe "DNAΣ"

5'TAATACGACTCACTATAGGGCGGGACAAGAAGGTGGAAGACGTCATG CTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTTCTTCGACTCACTG CAGACTACTGATGGAATGACGTAGTACGAATACTCGACTGGTCTCAACA TGAAAAAAAAAAAAAAACGCATTCAACCTGTCTGACTA3' (SEQ ID No 7).

TAATACGACTCACTATAGGG = sequence of the T7 promoter (SEQ ID No 8)

CGGGACAAGAAGGTGGAAGACGTCATG = 27 bp insert (SEQ ID No 9)

CTCCCGGCCGCCATGGCGGCCGCGGGAATTCGAT = 34 bp from pGEM®-T Easy sequence (SEQ ID No 10)

TTCTTCGACTCACTGCAGACTACTGATGGAATGACGTAGTACGAATACT
CGACTGGTCTCAACATGAAAAAAAAAAAAAAACGCATTCAACCTGTCTGA
CTA = 101 bp insert (SEQ ID No 11).

Example 2: *In vitro* transcription of the synthetic mRNA using the plasmid pGEM®-DNAΣ

- Linearization of the plasmid pGEM®-DNAΣ with the BSM I restriction enzyme (New England BioLabs®*Inc)*; see Figure 1.

- *In vitro* transcription with T7 RNA polymerase using T7-MEGAshortscriptTM (Ambion ®).

5'GGGCGAAUUGGGCCCGACGUCGGGACAAGAAGGUGGAAGACGUCAU
GCUCCCGGCCGCCAUGGCGGCCGCGGGAAUUCGAUUUCUUCGACUCAC
UGCAGACUACUGAUGGAAUGACGUAGUACGAAUACUCGACUGGUCUC
AACAUGAAAAAAAAAAAAAAA 3' (SEQ ID No 1)

Synthetic poly A mRNA #1 (161 nt)

Example 3: *In vitro* transcription of the synthetic mRNA using the DNA probe « DNAΣ »

- Digestion of the DNA probe « DNAΣ » with the BSM I restriction enzyme (New England BioLabs®*Inc)*; see Figure 1.

- *In vitro* transcription with T7 RNA polymerase using T7-MEGAshortscript™ (Ambion ®).

5'UAAUACGACUCACUAUAGGGCGGGACAAGAAGGUGGAAGACGUCAU
GCUCCCGGCCGCCAUGGCGGCCGCGGGAAUUCGAUUUCUUCGACUCAC
UGCAGACUACUGAUGGAAUGACGUAGUACGAAUACUCGACUGGUCUC
AACAUGAAAAAAAAAAAAAAA 3' (SEQ ID No 2).

Synthetic poly A mRNA #2 (161 nt)

Example 4: RT-PCR of both synthetic mRNAs #1 and #2

4.1 Reverse transcription of synthetic poly A mRNAs #1 (SEQ ID No 1) and #2 (SEQ ID No 2) with oligo dT using M-MLV Reverse Transcriptase RNase H Minus, point mutant (Promega) leading to :

Synthetic cDNA #1
5'GGGCGAATTGGGCCCGACGTCGGGACAAGAAGGTGGAAGACGTCATG
CTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTTCTTCGACTCACTG
CAGACTACTGATGGAATGACGTAGTACGAATACTCGACTGGTCTCAACA
TGAAAAAAAAAAAAAA 3' (SEQ ID No 3).

and

Synthetic cDNA #2
5'TAATACGACTCACTATAGGGCGGGACAAGAAGGTGGAAGACGTCATG
CTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTTCTTCGACTCACTG
CAGACTACTGATGGAATGACGTAGTACGAATACTCGACTGGTCTCAACA
TGAAAAAAAAAAAAAA 3' (SEQ ID No 4).

4.2 Amplification of synthetic cDNAs #1 and #2 using PCR on the LightCycler® (Roche) using the QuantiTectTM SYBR® Green PCR kit (Qiagen) or the LC FastStart DNA Master SYBR Green I (Roche).

Primer III forward (19 nt): 5' CGG GAC AAG AAG GTG GAA G 3' (SEQ ID No 5).

Primer III reverse (22 nt): 5' AGT CTG CAG TGA GTC GAA GAA A 3' (SEQ ID No 6)

The amplified fragment from both synthetic cDNA #1 and cDNA #2 (82 pb) with primer pair III produces the following sequence:

5' GGGACAAGAAGGTGGAAGACGTCATGCTCCCGGCCGCCATGGCGGCC GCGGGAATTCGATTTCTTCGACTCACTGCAGACT 3' (SEQ ID No 14).

Example 5: Calibration of the RT reaction using SmRNAs of the invention in real-time PCR.

5.1 We tested several primers to optimize the sequence of the SmRNA as depicted above. The goal was to obtain segments within the sequence which are complementary to pairs of primers which will not dimerize during the reaction while being specific to the SmRNA.
Among the sequences tested, we show here below three examples illustrating the importance of the presence of such segments within the SmRNA of the invention.

Pair I: primer forward (20 nt) 5' AATTGGGCCCGACGTCGCAT 3' SEQ ID No 15 and primer reverse (20 nt) 5' CATGTTGAGACCAGTCGAGT 3' SEQ ID No 16

Pair II: primer forward (19 nt) 5' CGGGACAAGAAGGTGGAAG 3' SEQ ID No 17 and primer reverse (20 nt) 5' TCATGTTGAGACCAGTCGAG 3' SEQ ID No 18

Pair III: primer forward (19 nt) 5' CGGGACAAGAAGGTGGAAG 3' SEQ ID No 5 and primer reverse (22 nt) 5' AGTCTGCAGTGAGTCGAAGAAA 3' SEQ ID No 6

The left and right primers I, II and III were chosen to minimize as much as possible the PCR priming primer-dimers using the "primer 3" software.

Regarding primers I, control experiments without cDNA show the absence of any amplification, confirming the absence of primer dimerization (curve control, Figure 4). However, these primers did not provide satisfying specificity since (in absence of SmRNA during the RT), they recognized cDNAs obtained from hippocampal tissue (curve cDNA, Figure 4).

Based on these results, it was necessary to design different specific primers. Several specific software, including Primer3, did not allow us to design other primers matching the various cautious required such as the absence of self complementarity and melting temperatures. Therefore, we have inserted a 27 bp fragment in the pGEM®-T Easy Vector with a T4 DNA ligase (Promega) in order to design other primers.

The left and right primers II were chosen to minimize as much as possible the PCR priming primer-dimers. Control experiments without cDNA show the absence of any amplification, which confirms the absence of primer dimerization (curve control, Figure 5). However, the profile of the amplification curve of the pGEM®-DNAΣ, that the primer pair has a weak affinity for the sequence. Other specific primers have thus been designed.

In case of the primers III, control experiments, without cDNA containing the sequence to amplify, show the absence of any amplification, which confirms the absence of primer dimerization (curve control, Figure 6). Furthermore, the amplification curve shows that primers III have a high affinity for the sequence of the pGEM®-DNAΣ, which contains the sequence to amplify.

Using such tests, one can routinely obtain variants of the SmRNA of SEQ ID No 1 and corresponding primers optimized for use as mentioned in the present invention.

5.2 Calibration using primers III and the SmRNA of SEQ ID No 1.

5.2.1 Calibration of the amplification of the synthetic cDNA using the LightCycler® (Roche) and the QuantiTectTM SYBR® Green PCR kit (Qiagen).

Several dilutions of the purified synthetic cDNA were amplified with specific primer pair III and the crossing point was determined.

We evidence that the starting concentration of synthetic cDNA before amplification is linearly correlated with the value of the crossing point (validation #1, Figure 7).

5.2.2 Increasing amounts of synthetic mRNA #1 were reverse transcribed and amplified with specific primer pair III using QuantiTectTM SYBR® Green PCR kit (Qiagen) on the LightCycler®

We evidence that the amplification of the synthetic cDNA, following reverse transcription of the synthetic mRNA, is dependant of the concentration of the synthetic mRNA #1 reverse transcribed (validation #2, Figure 8).

5.2.3 Increasing amounts of synthetic mRNA #1 were reverse transcribed together with 500 ng of total hippocampus RNA of either control or pilocarpine-treated rats. Synthetic cDNA were amplified as above.

We evidence that the dilution of the synthetic mRNA #1 either within total tissue RNA from control or pilocarpine-treated rats does not affect the PCR efficiency (1.68) for synthetic mRNA #1 (validation #3, Figure 9).

5.2.4 Calibration of the amplification of the synthetic cDNA using the LightCycler® (Roche) and LC FastStart DNA Master SYBR Green I (Roche). Several dilutions of the purified synthetic cDNA were amplified and the crossing point was determined.

This calibration curve using the LC FastStart DNA Master SYBR Green I (Roche) is very similar to that obtained with the QuantiTect SYBR Green PCR kit (Qiagen) (see validation # 1, Figure 7), demonstrating that the synthetic cDNA can be equally amplified with the LightCycler using both kits (validation #4, Figure 10).

5.2.5 Calibration of the amplification of the synthetic cDNA using the LightCycler® (Roche) and LC FastStart DNA Master SYBR Green I (Roche). Several dilutions of the purified synthetic cDNA were amplified and the crossing point was determined. In this experiment, the serial dilutions have been performed with a constant concentration of cDNAs obtained from reverse transcription of total brain mRNAs, instead of water (validations # 1 and 4, Figures 7 and 10 respectively).

We evidence that the amplification of the synthetic cDNA is independent of the diluent used (water in validation # 4 = Figure 10 versus brain cDNAs in validation # 5 = Figure 11).

5.2.6 Illustration of the variability of the RT efficiency performed in 86 samples of total RNA from mouse olfactory bulb (OB).

500 ng of total RNA from 86 OB have been reverse transcribed together with 80 pg of SmRNA #1. Reverse transcription has been performed in a PCR Express Thermocycler (Hybaid). cDNA corresponding to the SmRNA #1 has then been quantitatively amplified with primer pair "III" using the LightCycler to control whether identical concentration of cDNA from SmRNA was present in each sample after the reverse transcription step.

Conclusion: The figure 12 shows a wide range of cDNA concentration after reverse transcription, indicating that the reverse transcription was not reproducible across the 86 samples.

5.2.7 Demonstration that SmRNA #1 is appropriate, instead of house-keeping gene GAPDH, to quantify specific mRNA following quantitative relative RT-PCR.

500 ng of total RNA from rats (control 2 month old; control 10 month old; DSP-4 treated 10 month old) have been reverse transcribed together with 80 pg of SmRNA #1. cDNAs corresponding to BDNF exon V, GAPDH and SmRNA have been

amplified using gene specific primers with the LightCycler. In each sample, BDNF was either corrected using either GAPDH value or SmRNA value.

Note that GAPDH cannot be used to normalize samples, since its expression is not constant among the three groups tested (Figure 13B). However, SmRNA appears to highlight differences that could not been evidenced before normalization of the reverse transcription (Figure 13A vs. Figure 13D). Furthermore, note that normalization with GAPDH induces biased conclusions (Figure 13B vs. Figure 13D).

5.2.8 Influence of the SmRNA #1 addition to the RT reaction the relative variation in the expression of specific genes.

Reverse transcription of 500 ng of total hippocampus RNA from either control or pilocarpine-treated rats. Piolcarpine treatment has been used to stimulate BDNF gene expression in the hippocampus. Exon 5 of the BDNF cDNA was amplified with specific primers and LC FastStart DNA Master SYBR Green I (Roche). BDNF mRNA concentration was quantified using PCR on the LightCycler and the standard curve equation: $y = -3.7621 x + 31.648$.

<u>BDNF mRNA concentration in</u>:

Control: 31.5 ± 3.7 A.U.

Pilocarpine: 219.9 ± 36.9 A.U.

Pilocarpine = 6.98 X control

Reverse transcription of 500 ng of total hippocampus RNA from either control or pilocarpine-treated rats, together with 80 pg of synthetic mRNA #1 added to the RT reaction mix. Exon 5 of the BDNF cDNA was amplified with specific primers and LC FastStart DNA Master SYBR Green I (Roche). BDNF mRNA concentration was quantified using PCR on the LightCycler and the standard curve equation: $y = -3.7621 x + 31.648$.

Synthetic cDNA was amplified with specific primers and QuantiTect SYBR Green I PCR kit (Qiagen). Synthetic mRNA #1 concentration was quantified using PCR on the LightCycler and the standard curve equation: $y = -4.717 x + 33.569$.

BDNF mRNA concentration in:

Control: 31.4 ± 1.1 A.U.
Pilocarpine: 245.5 ± 26.2 A.U.

Synthetic mRNA #1 concentration in:

Control: 1.627 ± 0.062 A.U.
Pilocarpine: 1.821 ± 0.053 A.U.

BDNF mRNA / synthetic mRNA in:

Control: 19.3
Pilocarpine: 134.8
Pilocarpine = 6.98 X control

Conclusion:

The addition of the synthetic mRNA to the reverse transcriptase (RT) reaction mix does not interfere with the reverse transcription of endogenous mRNA.

The normalization of BDNF cDNA by the synthetic cDNA allows to recover the same induction profile in pilocarpine-treated rats than that observed without the addition of the synthetic mRNA to the RT reaction mix.

Therefore, we conclude that the addition of the SmRNA of the invention into the RT reaction mix allows a reliable normalization of the RT reaction between samples without competition with target mRNAs.